

New Methods for Protein Analysis Using Ultra Violet/ Visible Spectroscopy

Ngoc Dung Thanh
Energy Research Undergraduate Laboratory Fellowship
University of Colorado at Boulder
National Renewable Energy Laboratory
Golden, Colorado

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Participant:

Research Advisor:

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Abstract

**New Methods for Protein Analysis Using Ultraviolet/Visible Spectrometer.
NGOC DUNG THANH (University of Colorado at Boulder, Boulder,
Colorado 80309) BONNIE HAMES (National Renewable Energy
Laboratory, Golden, Colorado 80401)**

Some waste biomass is becoming more useful in biomass to ethanol conversion. We are trying to understand the composition of biomass and how it varies naturally. Thus, we need protein analysis to better understand the process that converts biomass into ethanol. The Biofuels Ethanol Program wants to be able to monitor protein content in biomass and track it through ethanol conversion process. Corn stover, a waste biomass, is used in this analysis. Lignin is one of the substances that give corn stover its structure and it is toxic to all the organisms currently used to ferment sugars to ethanol. And so, we would like to be able to answer, does the presence of protein in the liquid process stream interfere with lignin measurements? Lignin was measured using UV/visible-Spectroscopy. Amino acids, the bases of protein were also measured using UV/visible spectroscopy. Then lignin and amino acids spectrums were compared for interference. Last but not least, a new method for protein analysis was developed.

Category: Chemistry

School Author Attends: University of Colorado at Boulder
DOE National Laboratory Attended: National Renewable Energy Laboratory
Mentor's Name: Bonnie Hames
Phone: 303-384-6345
E-Mail: Bonnie_Hames@nrel.gov

Author's Name: Ngoc Dung Thanh
Mailing Address: 3582 West 112th Circle
City/State/Zip: Westminster, CO 80031
Phone: 303-466-0334 or 720-341-6433
E-mail: ngoc.thanh@colorado.edu

Introduction

Over the last several years the Biofuels Program has increased its emphasis on speeding deployment and commercialization of Biofuels Technologies. Consistent with this objective, the Ethanol Project is interested in utilizing corn stover as a feedstock for near-term, large-scale bioethanol production. But why is corn stover the targeted biomass for feedstock? The answer lies in its corn kernels. Since corn kernels have been the largest substrate for current large-scale fuel and industrial ethanol production, corn stover, consisting of the stalks, leaves, and cobs of harvested corn plants, can also be made a potential co-substrate in existing corn-to-ethanol facilities. Critically important to process economics, ethanol yield is strongly influenced by feedstock composition, for even the smallest changes in feedstock composition can significantly affect bioethanol production costs.

Previously, a method for analyzing lignin in hydrolysis liquors, one of the components of corn stover, was developed for the study of composition of wood. Using the Ultra violet – visible (UV-Vis) spectrometer, an approximation of the amount of lignin was measured from these liquor samples. Unlike wood, corn stover contains both lignin and protein. Accordingly, the Biomass Analysis Team is currently trying to determine if the presence of protein in the liquid process stream interferes with lignin measurements. They are also concerned with how the amount of protein in corn stover affects the ethanol conversion yield. This project has been proposed to develop a UV-vis method for monitoring protein content in biomass so that the effect of protein on ethanol production can be more accurately monitored. Ultimately the goal is to develop a new method for measuring protein in hydrolysis liquors.

Methods and Materials

Lignin Method: Samples of hydrolysis liquors were diluted until the absorbance of the maximum peak (180-210 nm) was between 0.7 and 1.0 where the concentration is known to be proportional to absorbance. For these samples, 20 μ l of hydrolysis liquor was dissolved in 10ml water.

UV-vis Spectroscopy: A UV-vis spectrum was collected from 190 nm to 350 nm, using a Hewlett Packard 845x UV-Visible spectrophotometer model 8453 with a diode array detector. The spectroscopic data was converted to spreadsheet form, which made it easy to view the spectrum graphically and to perform calculations from the absorbance data.

A full spectrum of the hydrolysis liquor was stored for comparison with the protein and amino acid spectra.

The UV-vis spectrum of each liquor sample was recorded within two hours of the sample generation to prevent loss of acid soluble lignin to flocculation reactions. Water was used as a background reference. The cuvettes used were high purity quartz with a 1 cm path length.

Amino Acids Procedure: Solutions of individual amino acids were prepared with a starting concentration of approximately 0.1 g/L. This starting solution was then diluted to provide a series of standard solutions with concentrations ranging from 0.1 g/L to 0.005 g/L. A dilution factor was calculated by dividing the total diluted volume by the original volume. The UV-vis spectrum of each amino acid sample was taken and the lambda max and the wavelength of each peak in the UV-vis spectrum were recorded. For each amino acid a wavelength was selected for use in the new method. The absorbance at the reference wavelength for each standard solution was measured and recorded. In an Excel worksheet, the concentration of each solution vs. the absorbance was plotted to reveal the linear region. If necessary, more dilutions of the standard

solutions were made to get at least six linear points. The linear regression was calculated for the linear region of each amino acid and the slope of that line, the extinction coefficient, was recorded.

Standard Coomassie method for Protein Determination (reference source kit #)

Materials:

1. Hewlett Packard 845x diode array UV-Visible spectrophotometer model 8453.
2. High purity quartz cuvettes with a path length of 1 cm
3. Automatic pipettes with adjustable volumes ranging from 0.1 to 10.0 mL.
4. Amino Acids: alanine, cysteine, aspartic acid, glutamine, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, arginine, serine, threonine, tryptophan, valine, tyrosine – all are Sigma grade.
5. Hydrolysis liquors: hydrolysis liquors from corn stover samples that varied in protein and lignin content. These samples were provided by a separate feedstock variability study.

Results

Lignin Measurements

The UV-vis spectrum of hydrolysis liquor spectra generally showed the highest absorbance, at wavelengths around 198nm (lambda max) and the next highest absorbance peak was located around 240nm. A typical spectrum is shown in Figure X. The absorbance of each sample at 198nm and 240 nm was recorded in an Excel worksheet where the concentration of lignin in each hydrolysis liquor sample was calculated using the known extinction coefficients for lignin at 198 nm and 240 nm and the following formula:

$$\text{concentration} = (\text{absorbance} * \text{dilution}) / (\text{extinction coefficient} * \text{path length})$$

The lignin concentration calculated from the absorbance at 198 nm was always higher than the lignin concentration measured at 240 nm as shown in Table 1.

Amino Acids Measurement

The UV-vis spectra of seventeen of the amino acids found in corn stover protein are shown in Figures 2 through 18. Most of the amino acids had similar spectral patterns, though each possessed a different maximum wavelength. The graph for each amino acid shows the absorbance changes with the series of dilutions. At high concentrations, the detector is saturated which gives the spectrum a jagged appearance. Next, a wavelength was selected, usually lambda max or the wavelength of a strong peak. This wavelength will be used for concentration calculations. The wavelengths for each amino acid are shown in Table 2. A graph was created plotting the absorbance at both lambda max and the selected wavelength for all dilutions. A concentration range was selected where the absorbance appeared to be linear with concentration according to Beer's law. The linear regression method was then used to find the slope of its

linear region, otherwise known as the extinction coefficient. These data were used as the basis for the new methods listed in the discussion section.

Comparison with Literature values and Standard Methods

The calculated extinction coefficients at lambda max were compared to literature values as shown in Table 3. Lastly, the absorbance at 240nm for concentration of about 0.1 g/L was recorded to determine its possible interference with the lignin measurements mentioned above. These absorbencies are shown in Table 4.

Standard Protein Analysis Method – Coomassie Plus Protein Assay Reagent Kit

Using a standard protein analysis protocol, developed for animal proteins, we calculated the concentration of soluble protein in the hydrolysis liquors. The concentrations of protein in the hydrolysis liquor samples from corn stover as determined using the Coomassie method are shown in Table 5.

Discussion and Conclusion

As seen in Figure 1, typical lignin spectra show absorbance peaks at 198 nm and 240 nm. The lignin concentration calculated at these two specific wavelengths was suspected to be high due to protein interference, especially at 198 nm since that concentration is higher than that calculated from the absorbance at 240 nm. As shown in Figure 2 through 18, none of these fifteen amino acids most abundant in corn protein in corn has any, absorbance at 240nm, but many have significant absorptions at 198nm. Therefore, lignin measurements at 240 nm have no significant protein interference. When analyzing biomass samples that contain protein, soluble lignin measurements should be made at 240 nm instead of 198 nm to minimize interference from protein.

Additionally, as demonstrated in Figure 1, there is no wavelength specific to amino acids in which there is no lignin or sugar degradation products interference. As a result, another conclusion drawn from this project is UV/Visible Spectroscopy may not be the best method for protein measurements in the presence of lignin and sugar degradation products.

New methods for calculating the concentration of each individual amino acid were written. These extinction coefficients were calculated using moles and were developed for the particular wavelength stated. Figures 19 through 35 show the absorbance versus concentration relationship for each of the 17 amino acids. A range of absorption was specified for each amino acid to assure that the concentration would be measured in a region where the absorbance was known to be proportional to concentration. These methods give concentration values in mole per liter. With this reference, protein estimates can be made from the same spectroscopic data used for lignin determinations.

New Methods for Calculating the Concentration of Amino Acids in Aqueous Solution

Dilute your sample until the concentration at 205 nm is between 0.0075 ml and 0.025 ml.
Calculate the concentration of Alanine using 22.2 as the extinction coefficient

Dilute your sample until the concentration at 194 nm is between 0.025 g/L and 0.075 g/L.
Calculate the concentration of Cysteine using 1105.3 as the extinction coefficient

Dilute your sample until the concentration at 198 nm is between 0.0075 g/L and 0.025 g/L. Calculate the concentration of Aspartic Acid using 254.6 as the extinction coefficient

Dilute your sample until the concentration at 206 nm is between 0.005 g/L and 0.03 g/L.
Calculate the concentration of Phenylalanine using 5835.2 as the extinction coefficient

Dilute your sample until the concentration at 201 nm is between 0.0075 g/L and 0.009 g/L. Calculate the concentration of Glycine using 467.0 as the extinction coefficient

Dilute your sample until the concentration at 211 nm is between 0.005 g/L and 0.015 g/L.
Calculate the concentration of Histidine using 4787.8 as the extinction

Dilute your sample until the concentration at 195 nm is between 0.025 g/L and 0.075 g/L.
Calculate the concentration of Isoleucine using 225.4 as the extinction coefficient

Dilute your sample until the concentration at 190 nm is between 0.005 g/L and 0.05 g/L.
Calculate the concentration of Lysine using 639.4 as the extinction coefficient

Dilute your sample until the concentration at 206 nm is between 0.005 g/L and 0.09 g/L.
Calculate the concentration of Leucine using 66.8 as the extinction coefficient

Dilute your sample until the concentration at 208 nm is between 0.005 g/L and 0.01 g/L.
Calculate the concentration of Methionine using 1509.4 as the extinction coefficient

Dilute your sample until the concentration at 198 nm is between 0.0065 g/L and 0.015 g/L.
Calculate the concentration of Proline using 173.8 as the extinction coefficient

Dilute your sample until the concentration at 220 nm is between 0.08 g/L and 0.1 g/L.
Calculate the concentration of Glutamine using 99.4 as the extinction coefficient

Dilute your sample until the concentration at 205 nm is between 0.0065 g/L and 0.01 g/L.
Calculate the concentration of Arginine using 1652.4 as the extinction coefficient

Dilute your sample until the concentration at 198 nm is between 0.0065 g/L and 0.01 g/L.
Calculate the concentration of Serine using 292.7 as the extinction coefficient

Dilute your sample until the concentration at 190 nm is between 0.005 g/L and 0.01 g/L.
Calculate the concentration of Threonine using 730.9 as the extinction coefficient

Dilute your sample until the concentration at 198 nm is between 0.005 g/L and 0.0085 g/L.
Calculate the concentration of Valine using 1269.9 as the extinction coefficient

Dilute your sample until the concentration at 220 nm is between 0.005 g/L and 0.0075 g/L. Calculate the concentration of Tryptophan using 28396.1 as the extinction coefficient. All calculations are made using the following equation:

$$\text{concentration} = (\text{absorbance} * \text{dilution}) / (\text{extinction coefficient} * \text{path length})$$

The wavelengths selected for our new methods and the extinction coefficients used are sometimes different from those reported in the literature. In many cases, the lambda max is near 200 nm or near the edge of the detector cut-off. Recent improvements in the detectors used in UV-vis spectrometers have affected the quality and reproducibility of measurements taken at lower wavelengths.

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- Sigma Chemical Company. Louis, Montana.

Table 1***Wisconsin Samples***

Sample Name	Sample #	Concentration at 198 nm	Concentration at 240 nm
2913-017	17	8.8	6.0
2913-017B	17B	7.8	5.5
2893-025	25	6.6	4.7
2893-025B	25B	11.3	7.5
2893-069	69	7.8	5.2
2893-069B	69B	8.2	5.7
2893-103	103	9.7	5.7
2893-103B	103B	9.9	6.0
2983-111	111	10.5	6.8
2893-111B	111B	7.0	5.1
2870-119	119	7.7	5.6
2870-119B	119B	7.2	5.3

Minnesota Samples

Sample Name	Sample #	Concentration at 198 nm	Concentration at 240 nm
2798-002	2	7.4	4.4
2798-002B	2B	8.3	4.8
2891-004	4	9.9	5.5
2891-004B	4B	10.7	6.1
2891-004C	4C	10.1	5.8
2891-004D	4D	10.4	5.7
2798-012	12	9.0	5.9
2798-012B	12B	9.1	5.8
2798-012C	12C	9.7	6.3
2798-012D	12D	8.8	5.7
2891-014	14	7.8	6.1
2891-014B	14B	6.8	5.3
2891-021	21	8.2	5.1
2891-021B	21B	8.6	5.4
2892-027	27	9.2	5.6
2892-027B	27B	8.6	5.1
2892-027C	27C	8.1	5.0
2892-027D	27D	8.9	5.7

Table 2

Sample Concentration		Substance	Extinction Coefficient [L/(mol*cm)]
Wavelength (nm)	Concentration Range		
205	0.0075 ml and 0.025 ml	alanine	22.2
194	0.025 g/L and 0.075 g/L	cysteine	1105.3
198	0.0075 g/L and 0.025 g/L	aspartic acid	254.6
206	0.005 g/L and 0.03 g/L	phenylalanine	5835.2
201	0.0075 g/L and 0.009 g/L	glycine	467
211	0.005 g/L and 0.015 g/L	histidine	4787.8
195	0.025 g/L and 0.075 g/L	isoleucine	225.4
190	0.005 g/L and 0.05 g/L	lysine	639.4
206	0.005 g/L and 0.09 g/L	leucine	66.8
208	0.005 g/L and 0.01 g/L	methionine	1509.4
198	0.0065 g/L and 0.015 g/L	proline	173.8
220	0.08 g/L and 0.1 g/L	glutamine	99.4
205	0.0065 g/L and 0.01 g/L	arginine	1652.4
198	0.0065 g/L and 0.01 g/L	serine	292.7
190	0.005 g/L and 0.01 g/L	threonine	730.9
198	0.005 g/L and 0.0085 g/L	valine	1269.9
220	0.005 g/L and 0.0075 g/L	tryptophan	28396.1

Table 3**Comparison Chart**

Amino Acid	Max Wav (lit)	Ext Coef (lit)	Extinction Coef (recorded at lit wavelength)
alanine	205	5	25
cysteine	231	3090	299.7
asparagine(aspartic Acid)	220	100	35.1
phenylalanine	257	178	179.1
glycine	285	5754	4.1
histidine	211	6310	4787.8
isoleucine	N/A	N/A	225.4
lysine	N/A	N/A	639.4
leucine	207	66	66.8
methionine	208	1585	1509.4
proline	N/A	N/A	173.4
glutamine	220	100	99.4
arginine	205	1905	1652.4
serine	N/A	N/A	292.7
threonine	N/A	N/A	730.9
Valine	N/A	N/A	2104.4
tryptophan	220	38800	28396.1
tyrosine	275	1413	

Table 4

Amino Acid Absorbencies at 240 nm

Amino Acid	Absorbance at 240 nm at .1 g/L
alanine	0.0014
cysteine	0.0281
asparagine(aspartic Acid)	0.0009
phenylalanine	0.0392
glycine	-0.0003
histidine	0.0032
isoleucine	0.0020
lysine	0.0013
leucine	0.0010
methionine	0.0151
proline	0.0006
glutamine	0.0187
arginine	0.0101
serine	-0.0001
threonine	0.0005
Valine	0.0010
tryptophan	0.8647
tyrosine	

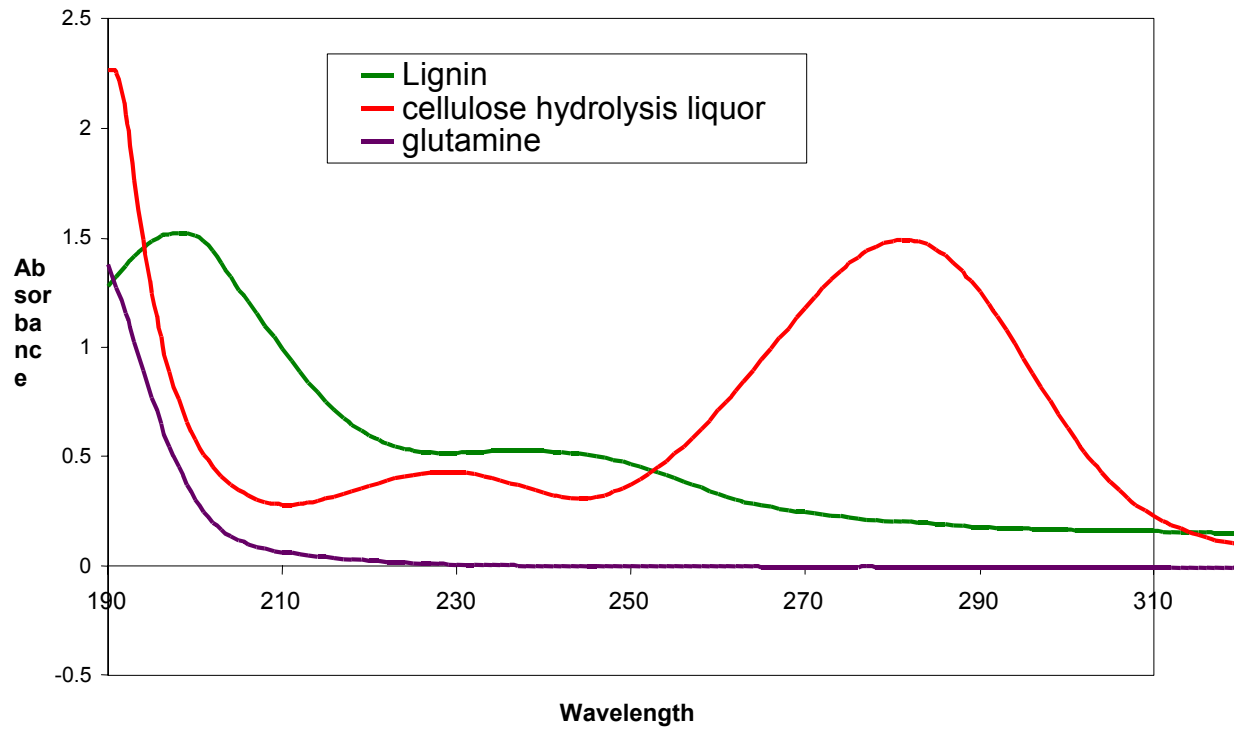
Table 5

Total Soluble Protein Concentration (g/L) Using Coomassie Method

Sample	Concentration (g/L)
2893-025	-0.01
2893-069	0.11
2893-103	0.24
2893-111	0.12
2870-119	0.03
2913-017	0.08
2798-002	0.09
2798-012	0.14
2891-004	0.13
2891-014	0.05
2891-021	0.05
2891-027	0.17
2893-025B	0.16
2893-069B	0.09
2893-103B	0.24
2893-111B	-0.01
2870-119B	0.00
2913-017B	0.07
2798-002B	0.09
2798-012B	0.16
2891-004B	0.14
2891-014B	0.04
2891-021B	0.06
2891-027B	0.16
2891-004C	0.14
2891-004D	0.12
2798-012C	0.15
2798-012D	0.13
2891-027C	0.12
2891-027D	0.14

Figure 1

Comparison Graph



General Spectrum of Amino Acids

Figure 2

Alanine

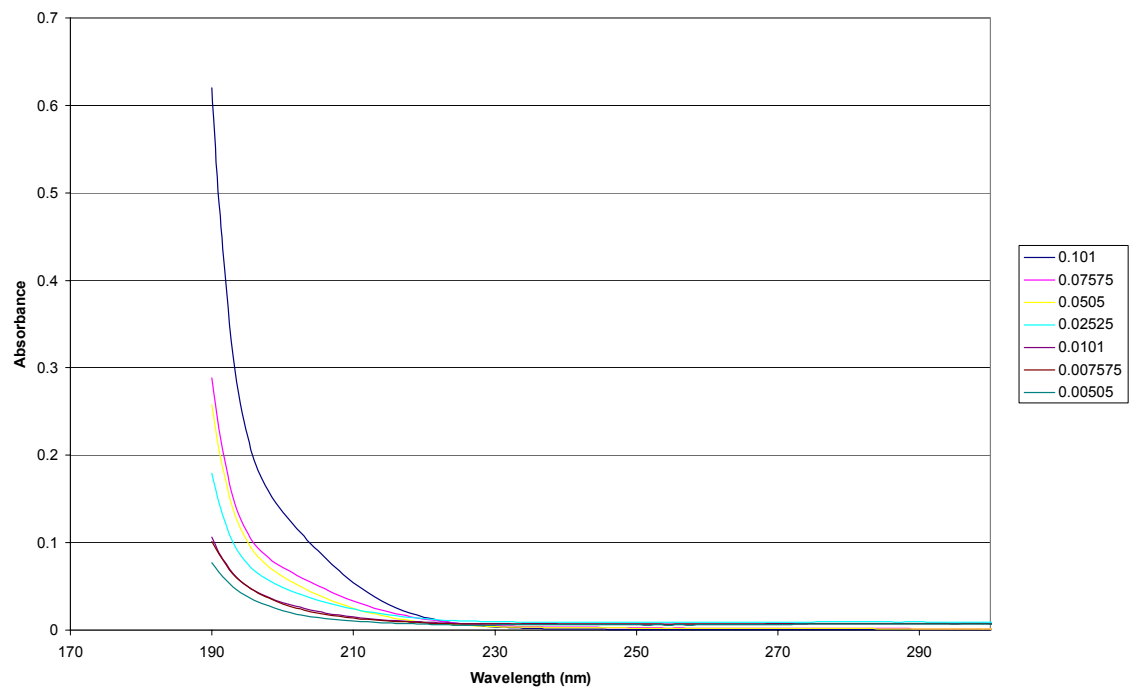


Figure 3

Cysteine

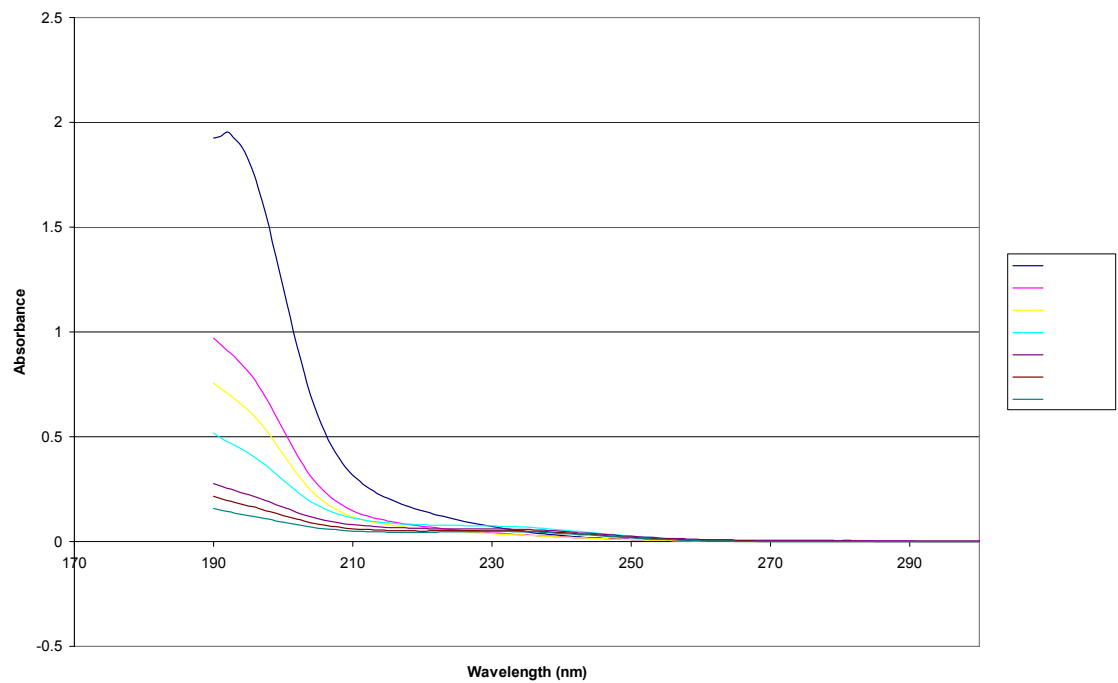


Figure 4

Aspartic Acid

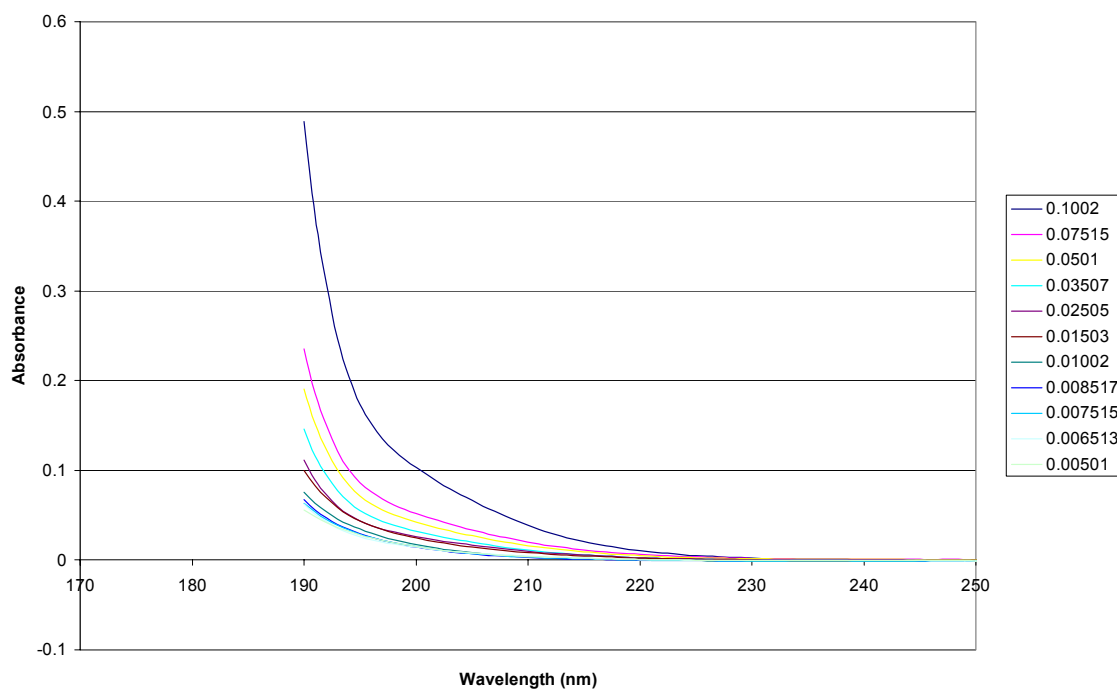


Figure 5

Phenylalanine

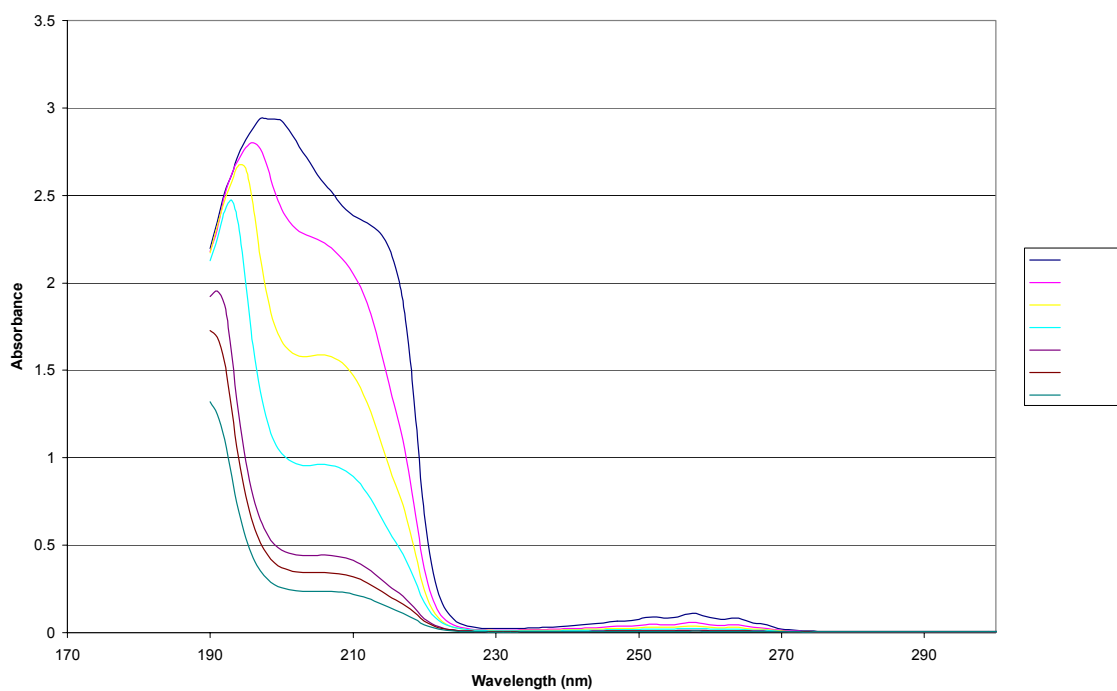


Figure 6

Glycine

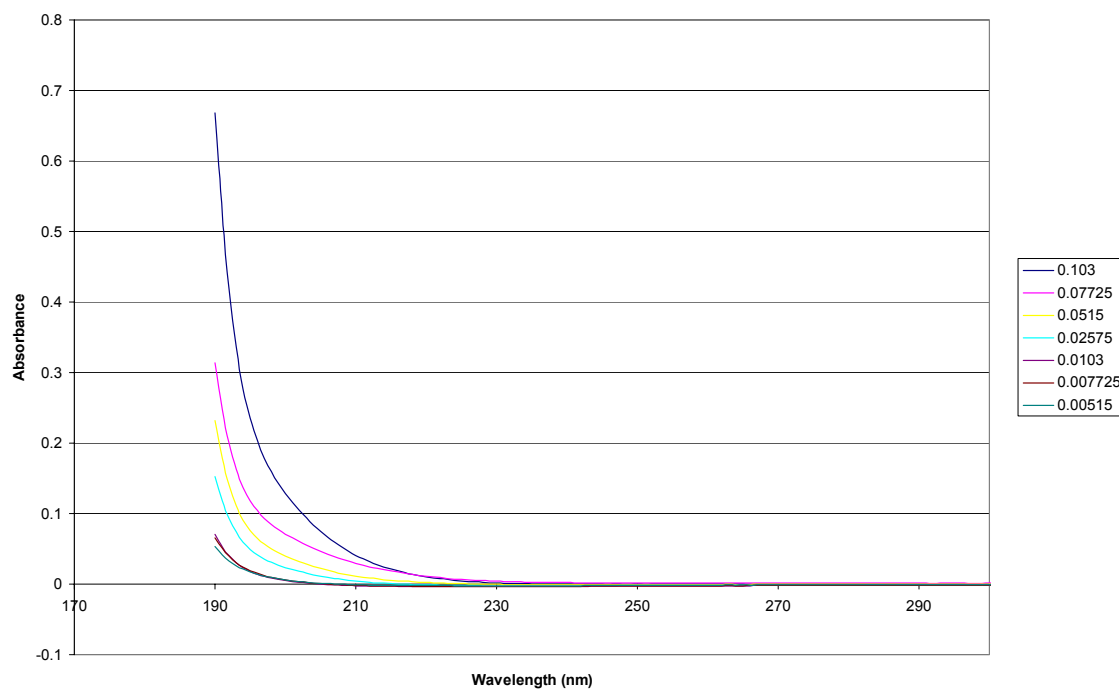
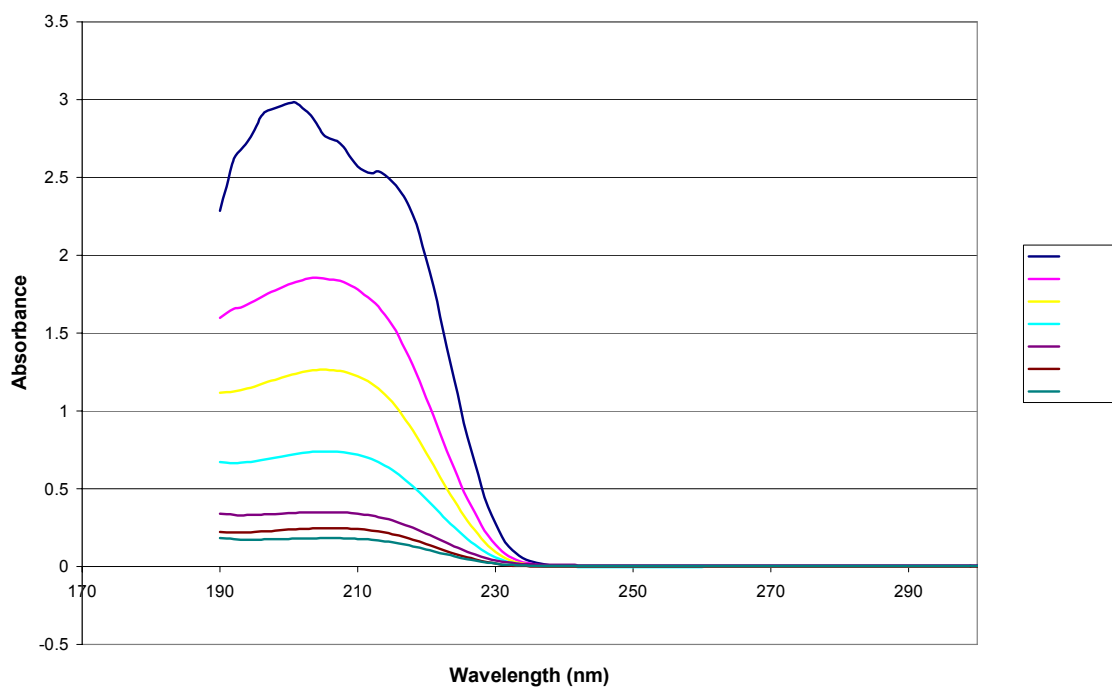
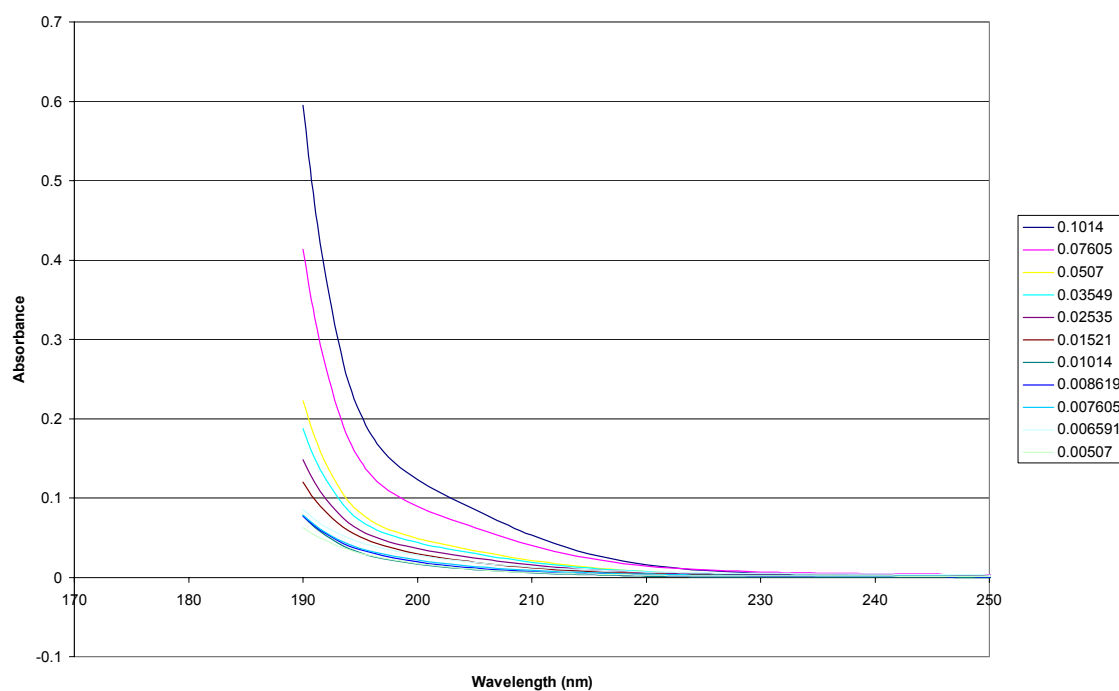


Figure 7

Histidine



Isoleucine



Lysine

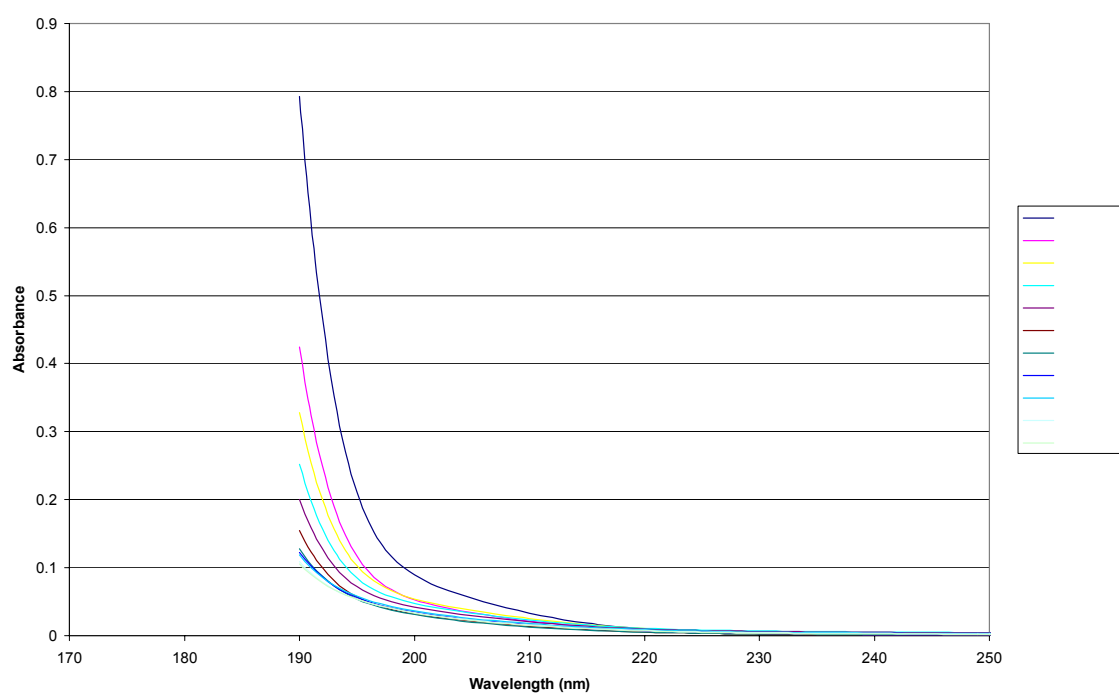


Figure 10

Leucine

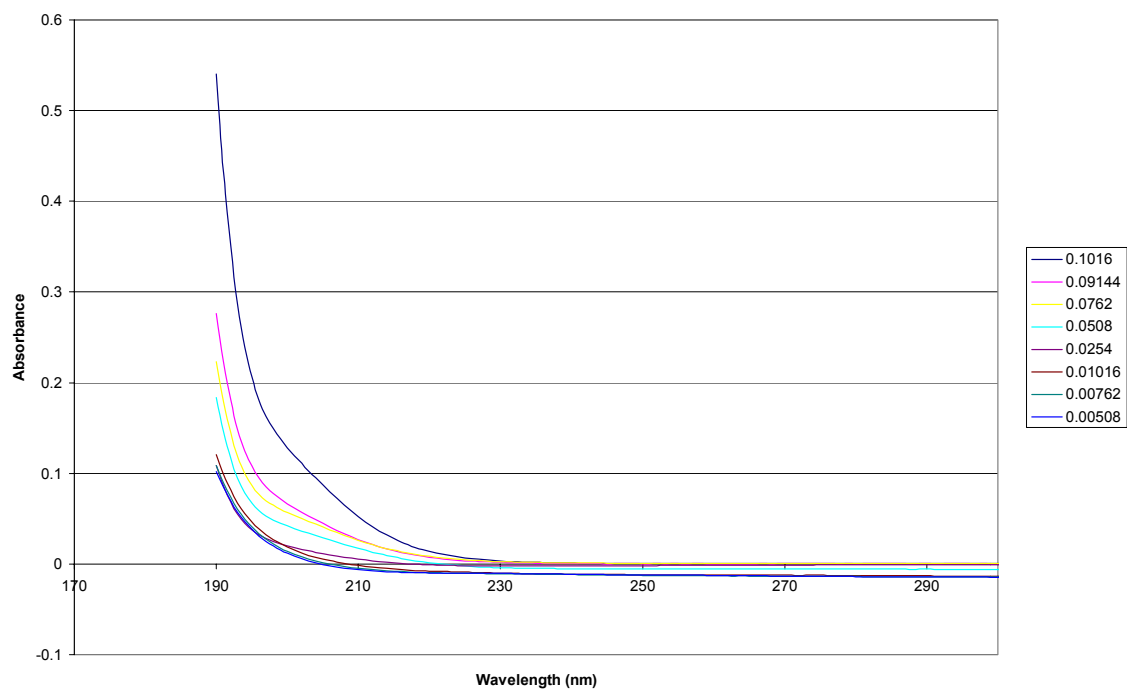


Figure 11

Methonine

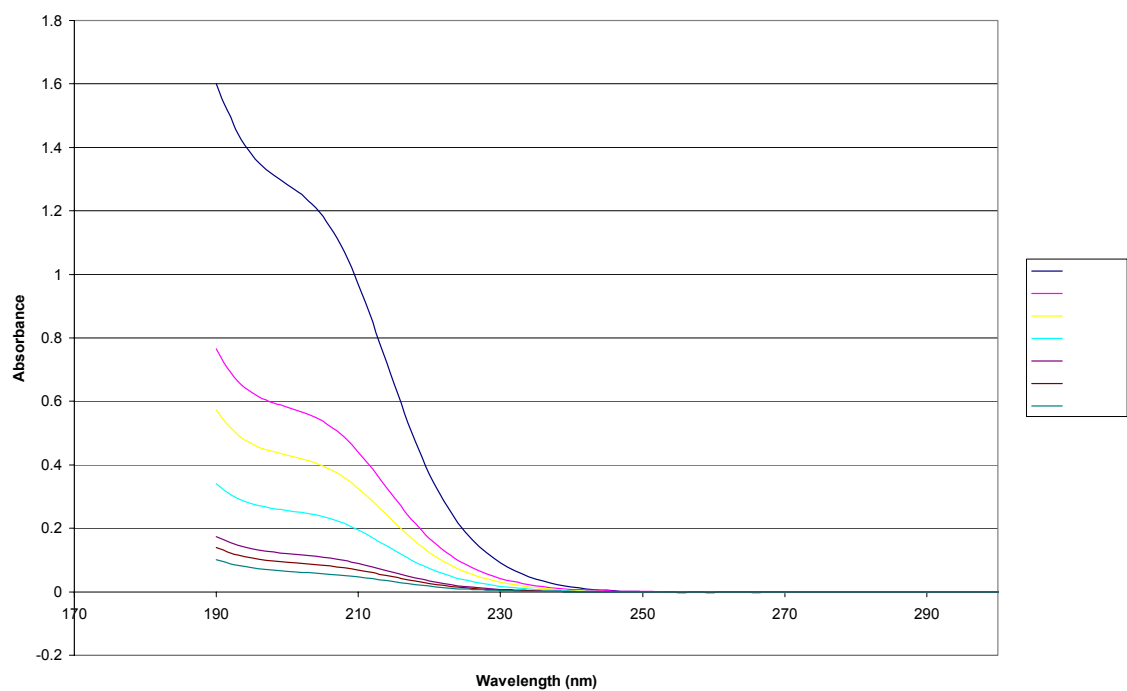


Figure 12

Proline

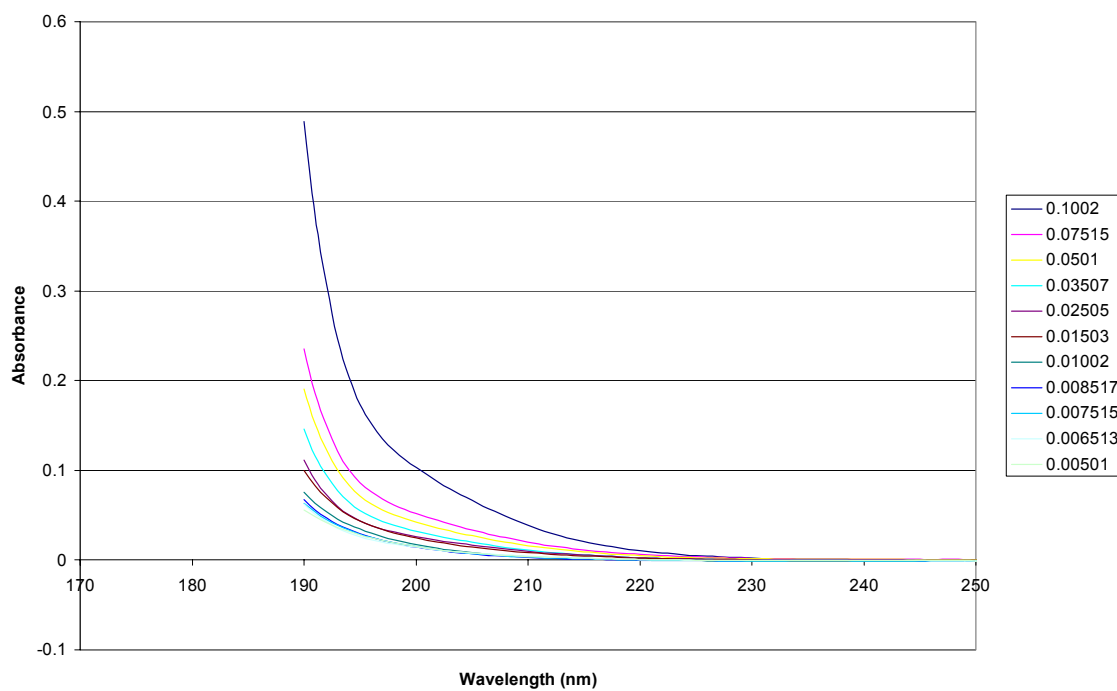


Figure 13

Glutamine

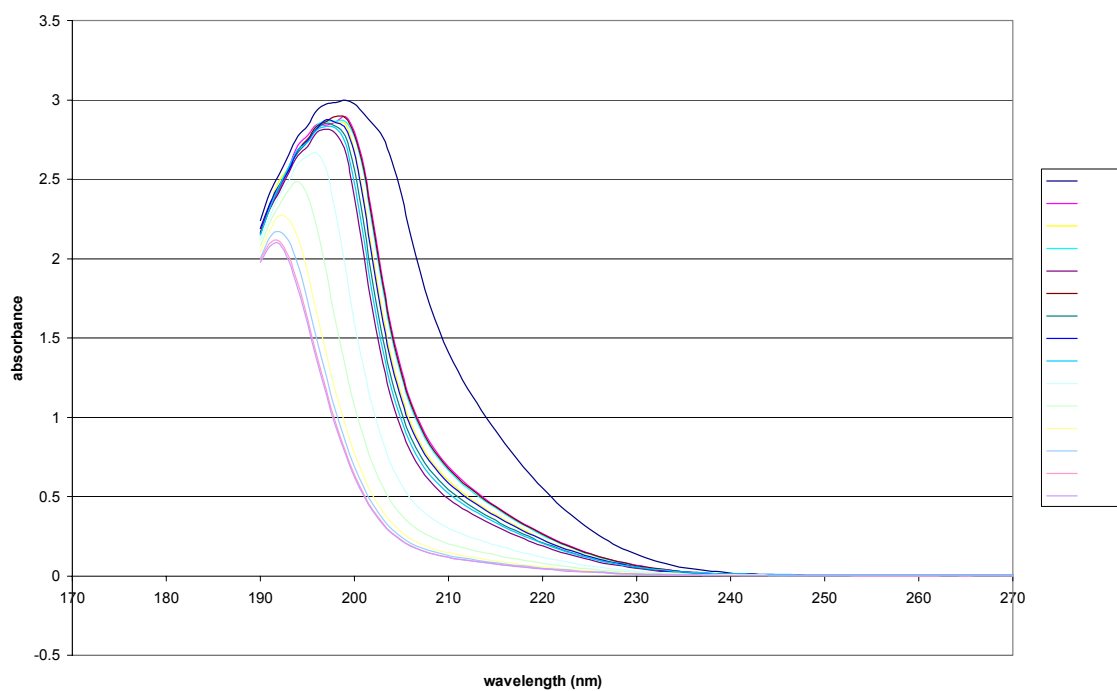


Figure 14

Arginine

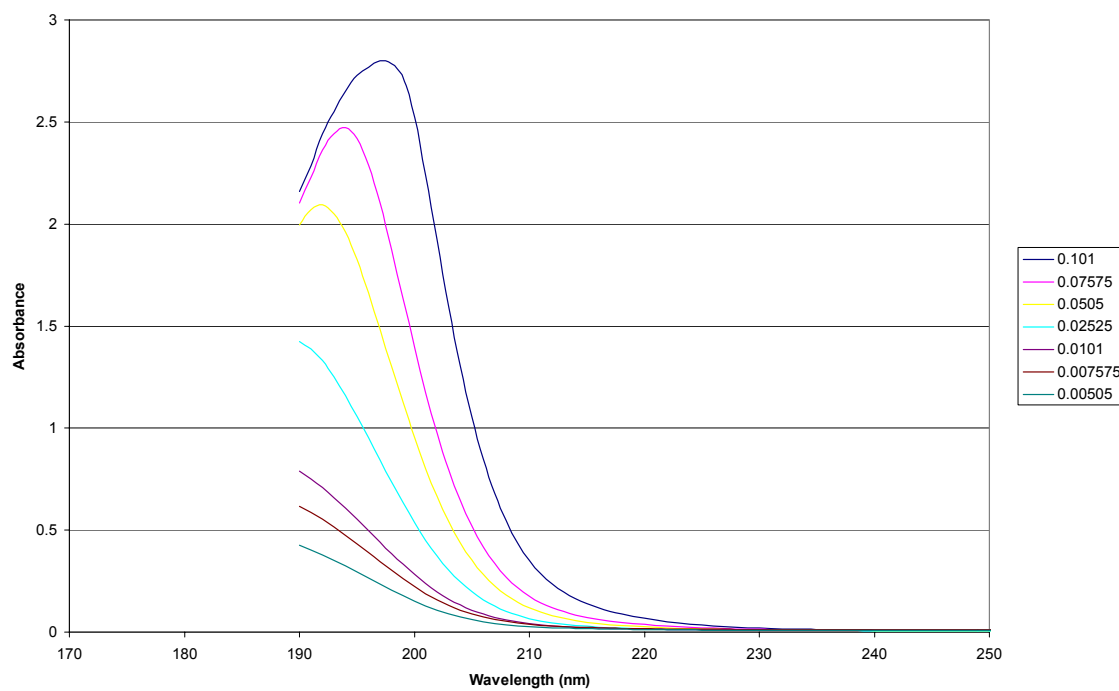


Figure 15

Serine

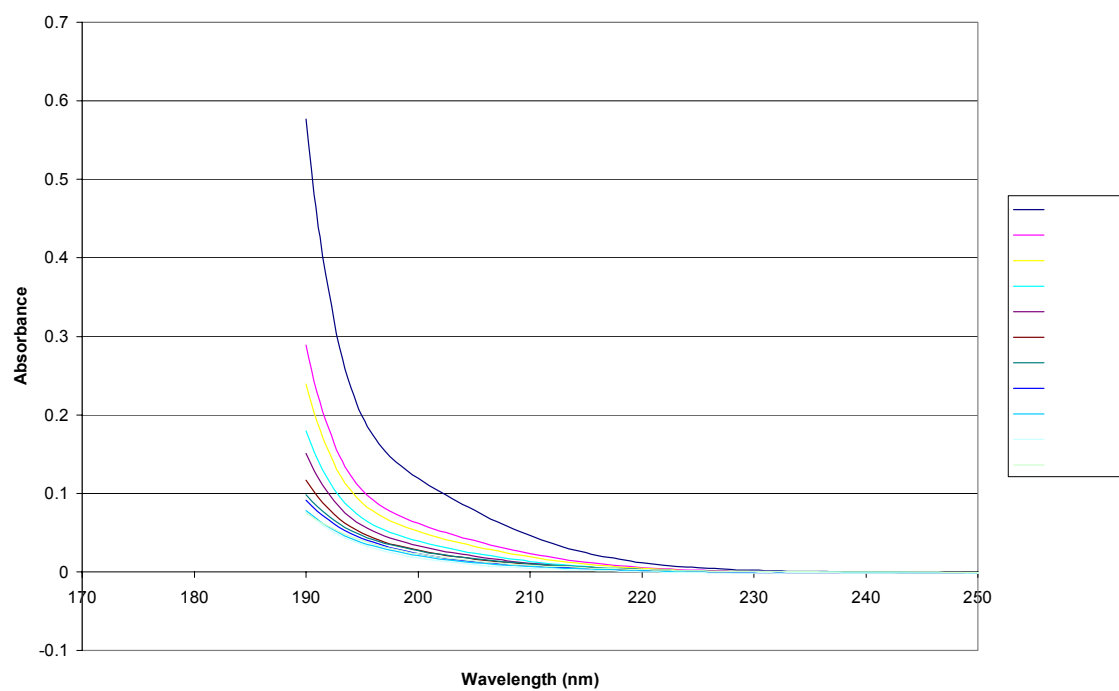


Figure 16

Threonine

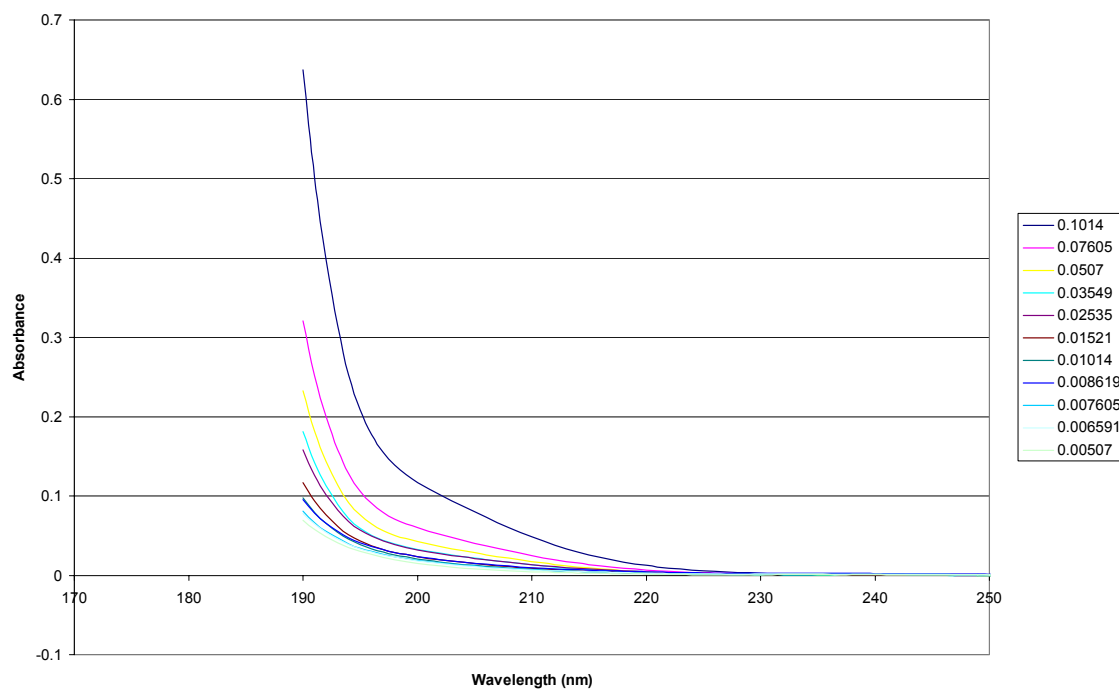


Figure 17

Valine

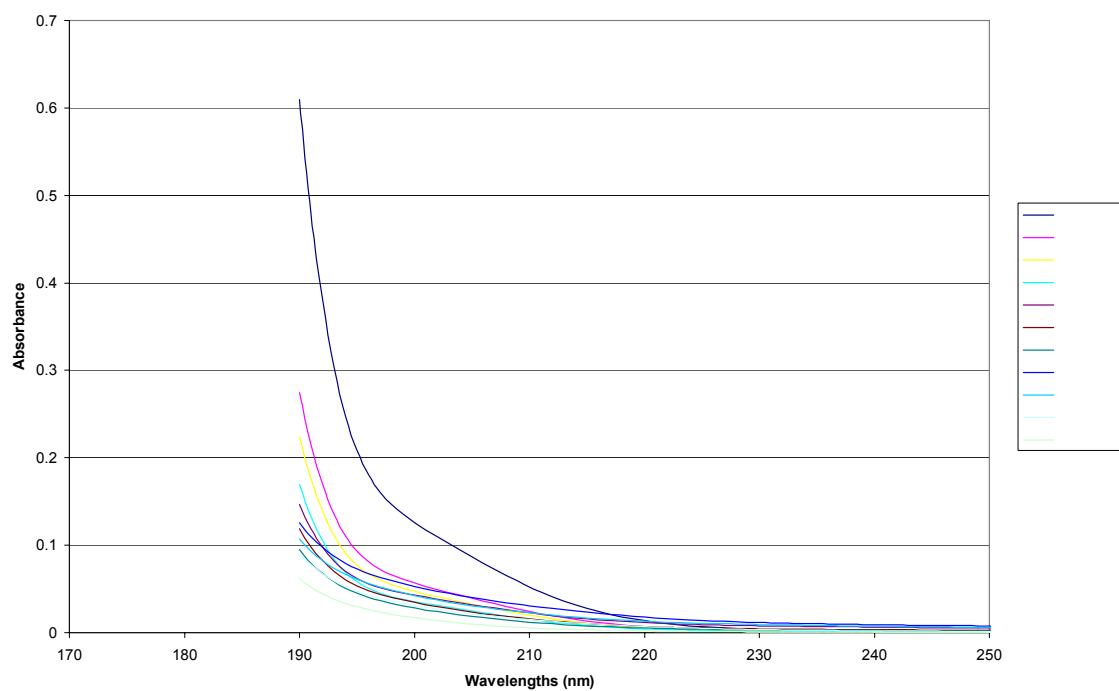
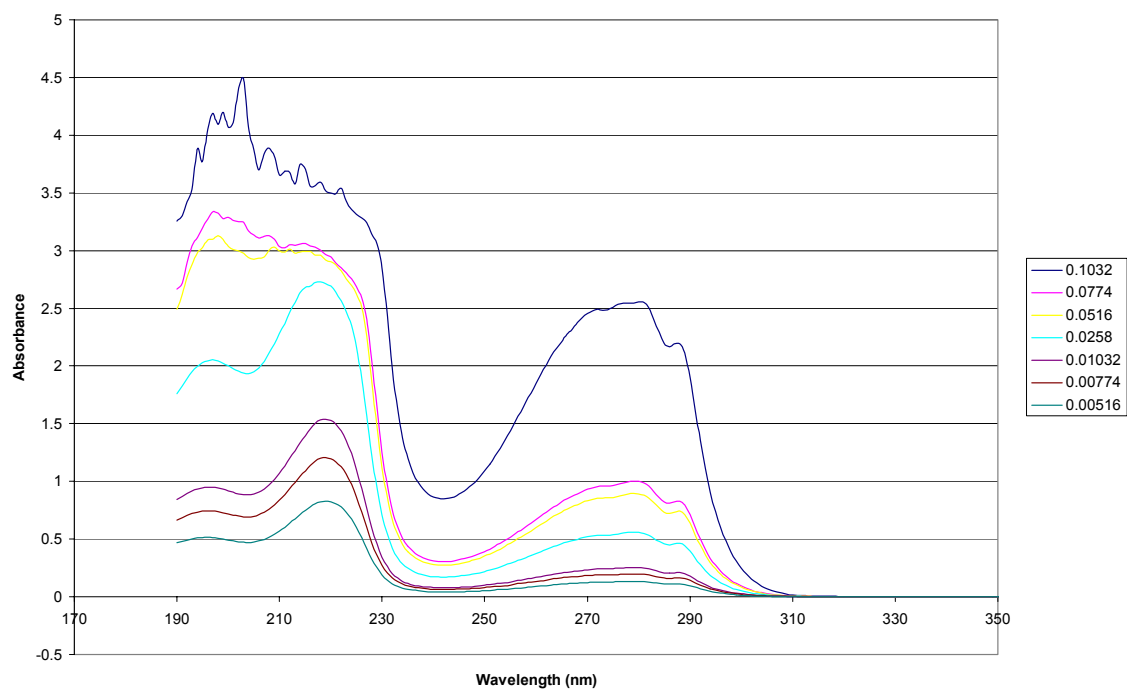


Figure 18

Tryptophan



Concentration vs. Absorbance Graph for Amino Acids at Specifically Chosen Wavelengths

Figure 19

Alanine: concentration vs. absorbance at 205 nm

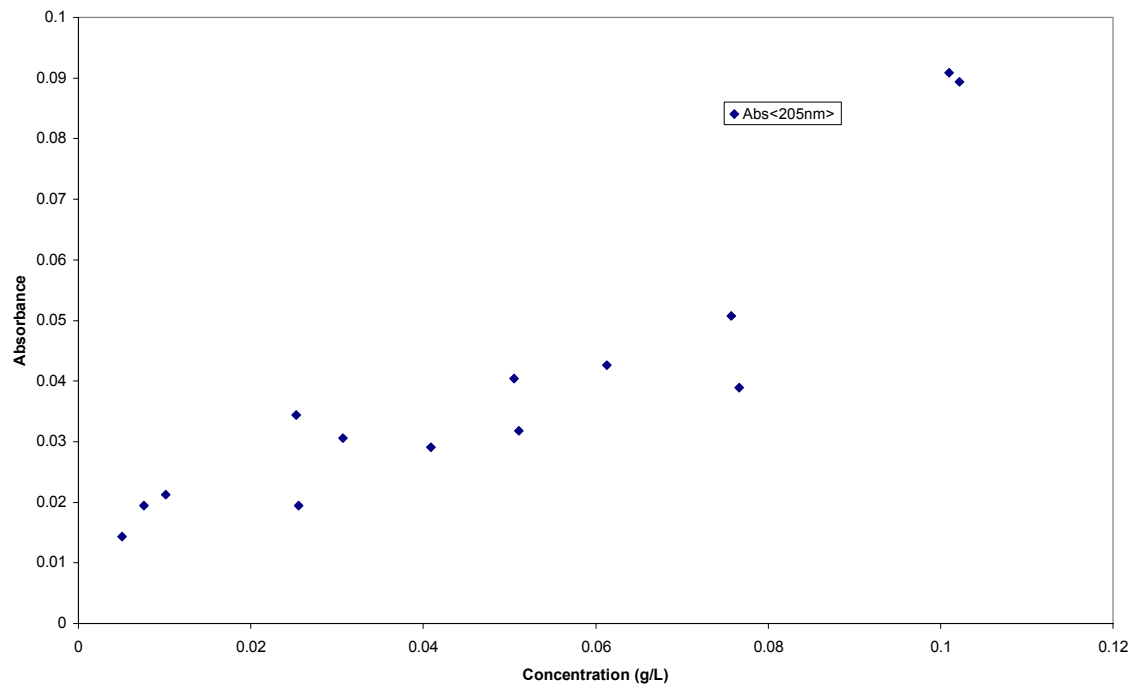


Figure 20

Cysteine: concentration vs. absorbance at 194nm

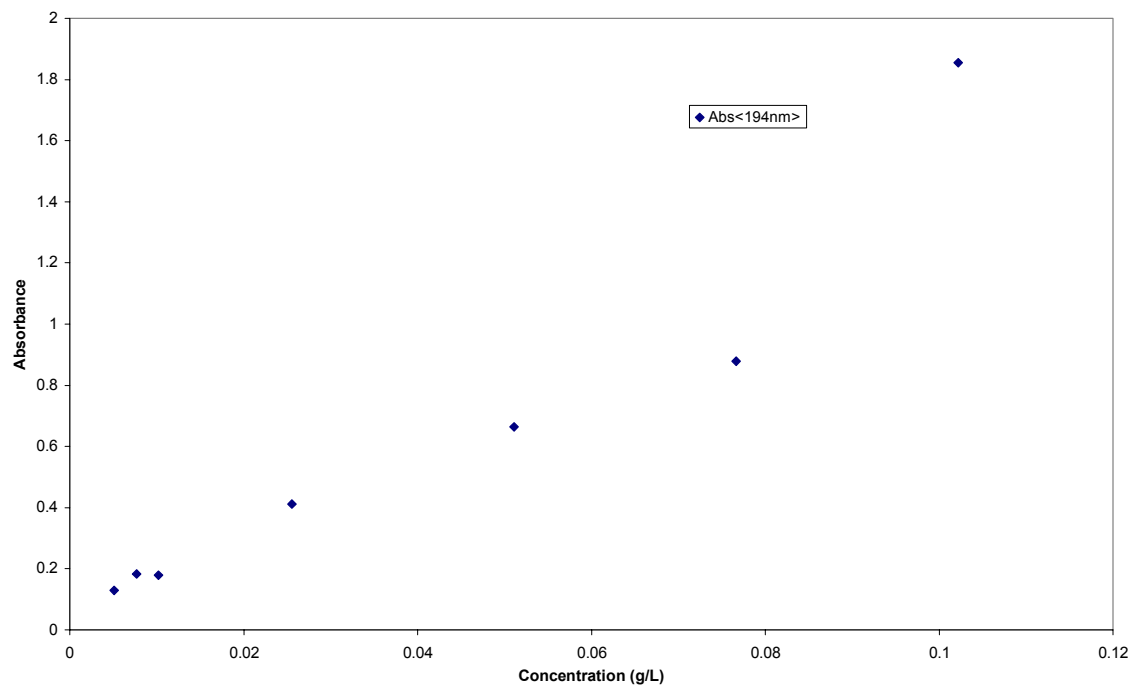


Figure 21

Aspartic Acid: concentration vs. absorbance at 198nm

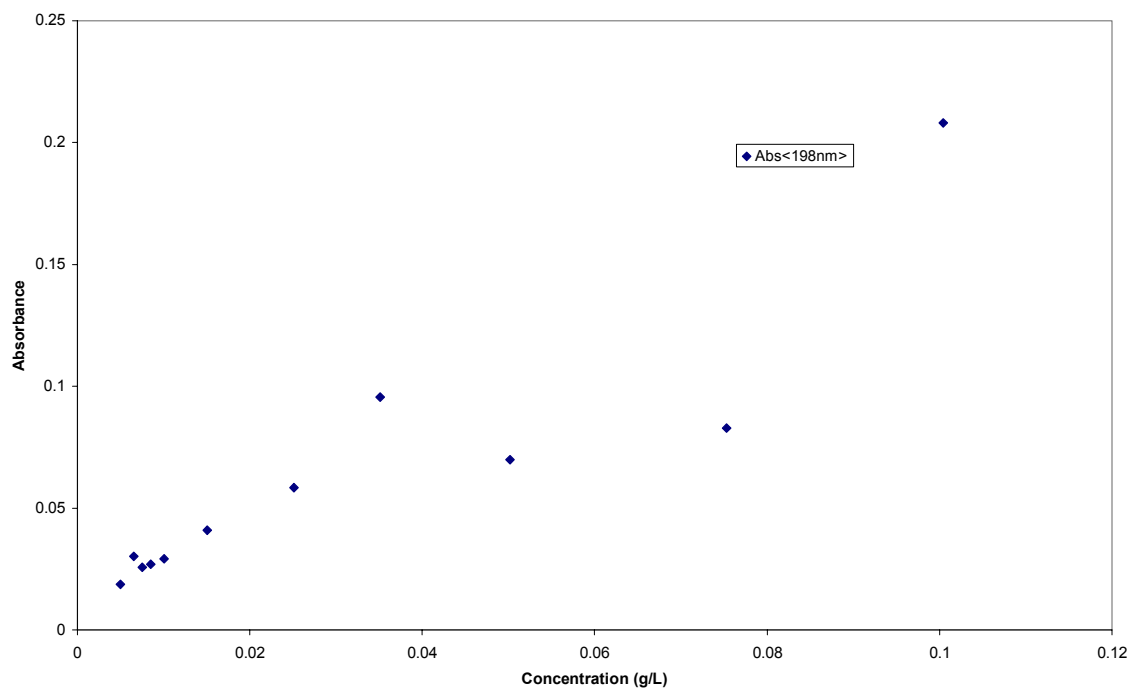


Figure 22

Phenylalanine: concentration vs. absorbance at 206 nm

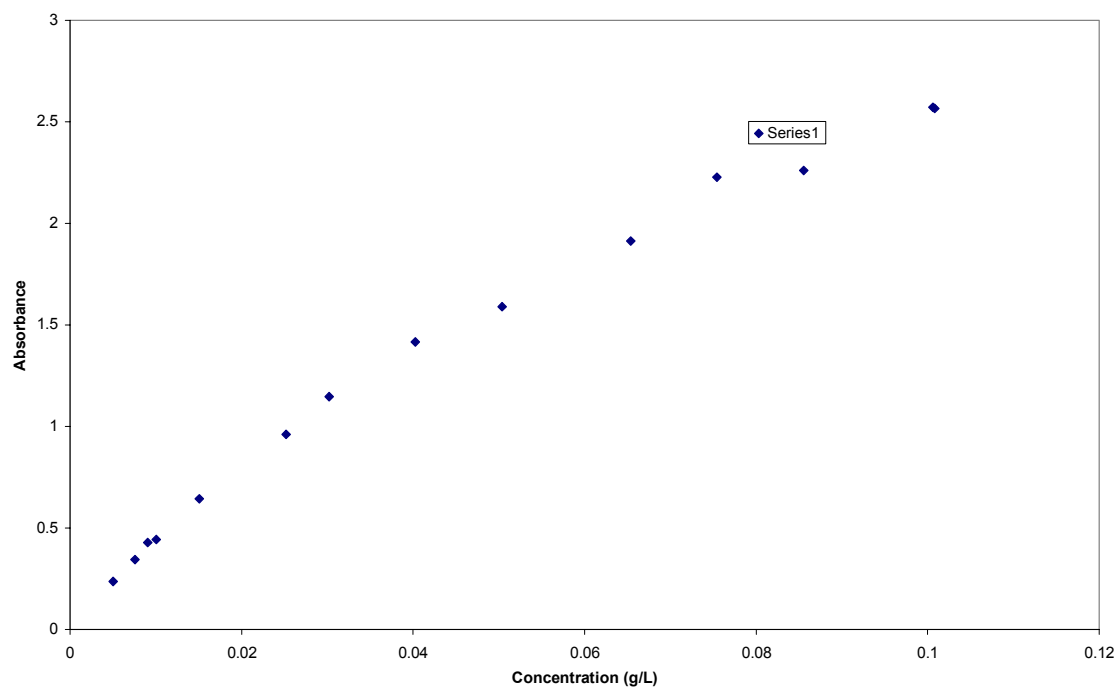


Figure 23

Glycine: concentration vs. absorbance at 201 nm

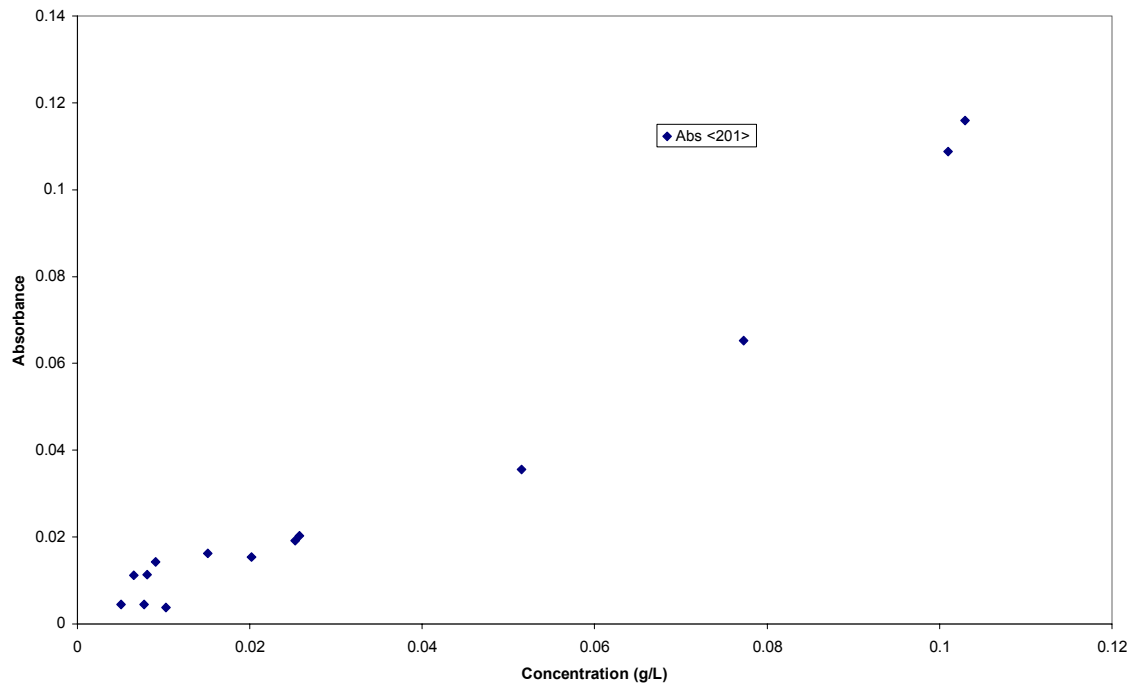


Figure 24

Histidine: concentration vs. absorbance at 211 nm

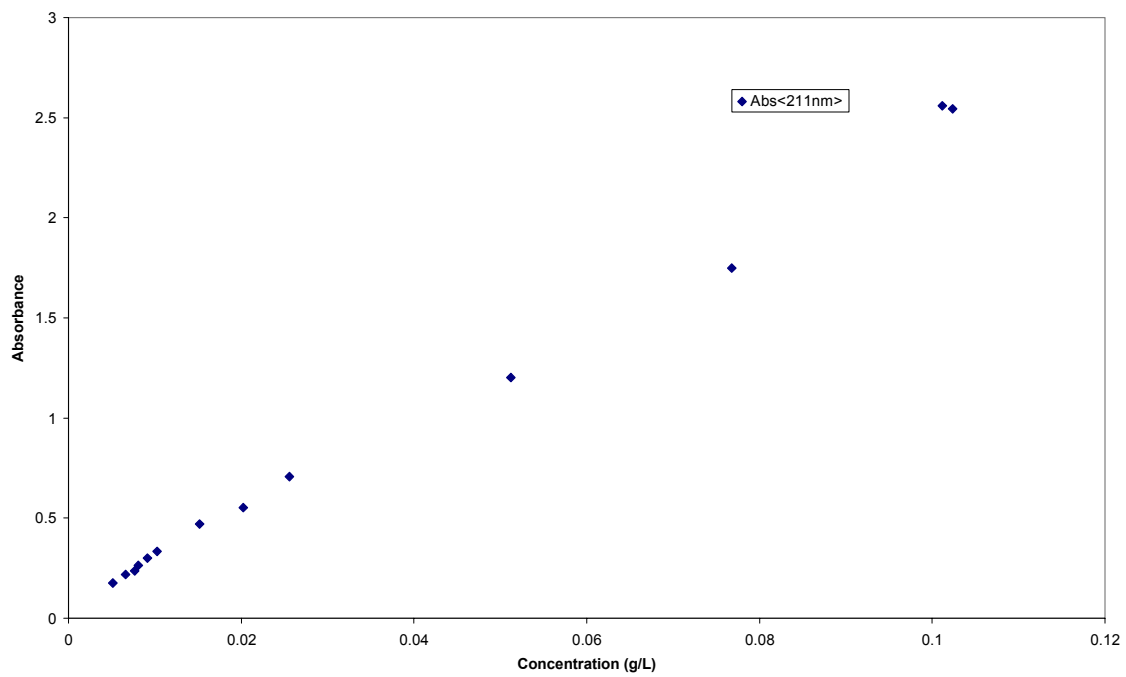


Figure 25

Isoleucine: concentration vs. absorbance at 195 nm

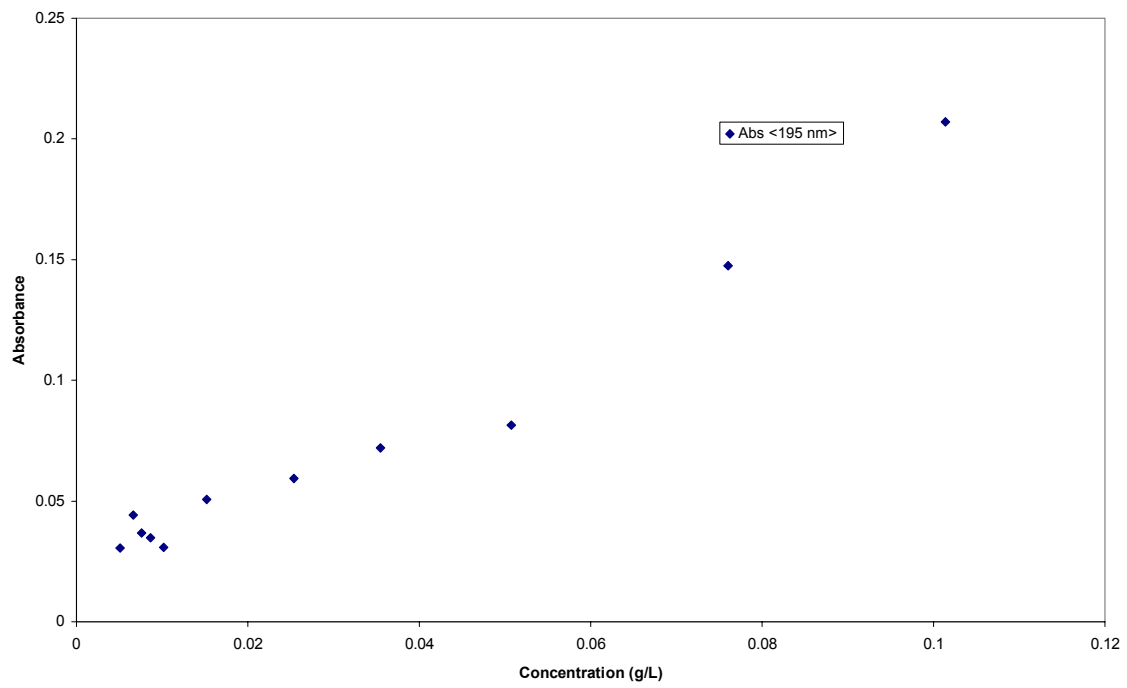


Figure 26

Lysine: concentration vs. absorbance at 190nm

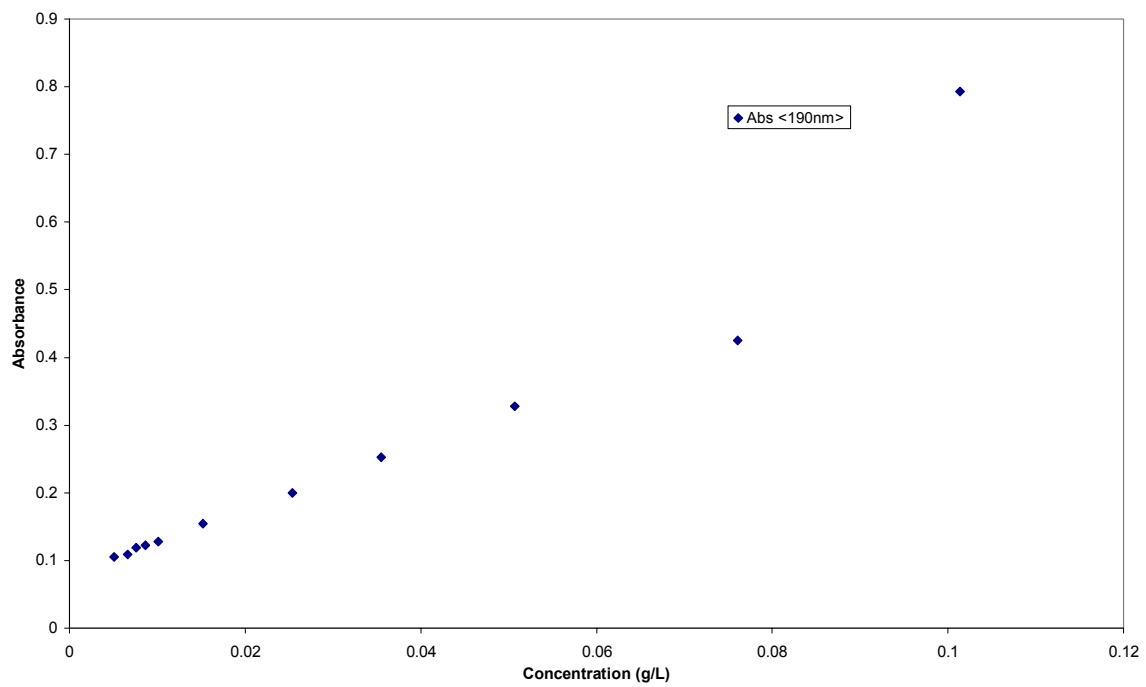


Figure 27

Leucine: concentration vs. absorbance at 206 nm

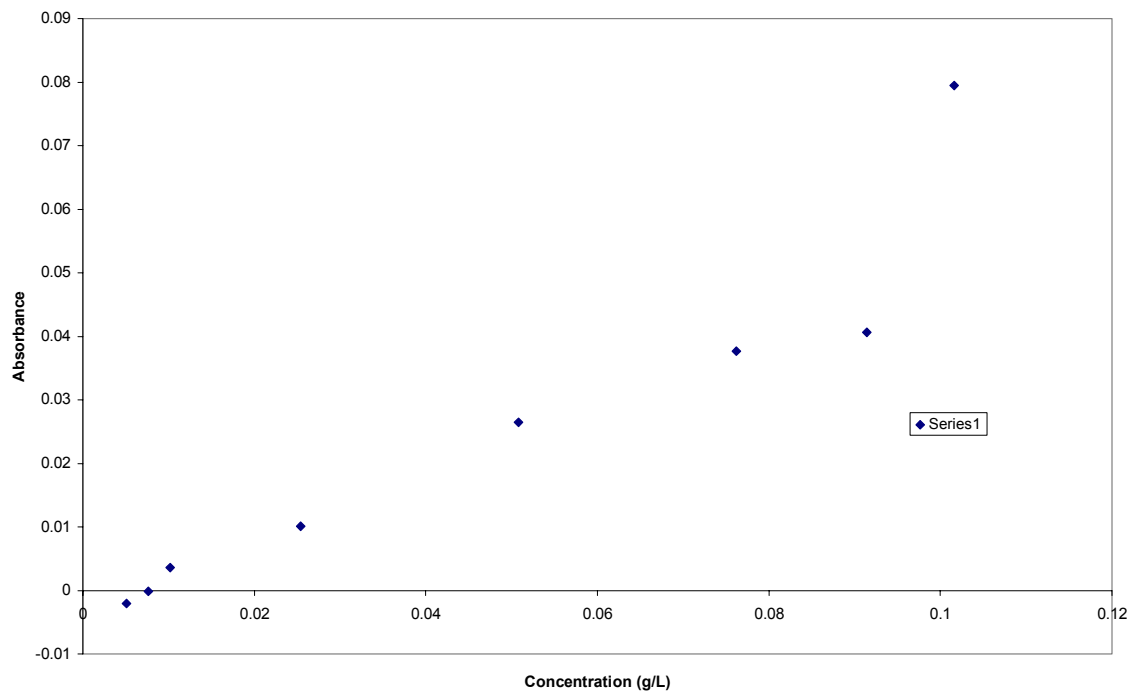


Figure 28

Methonine: concentration vs. absorbance at 208 nm

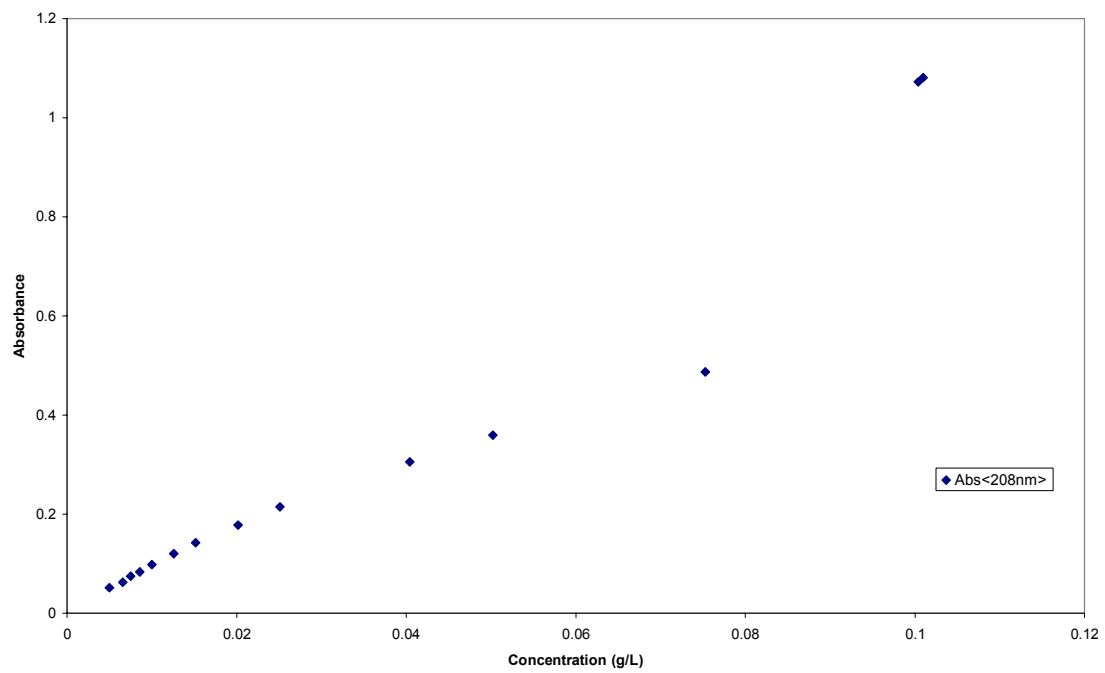


Figure 29

Proline: concentration vs. absorbance at 198nm

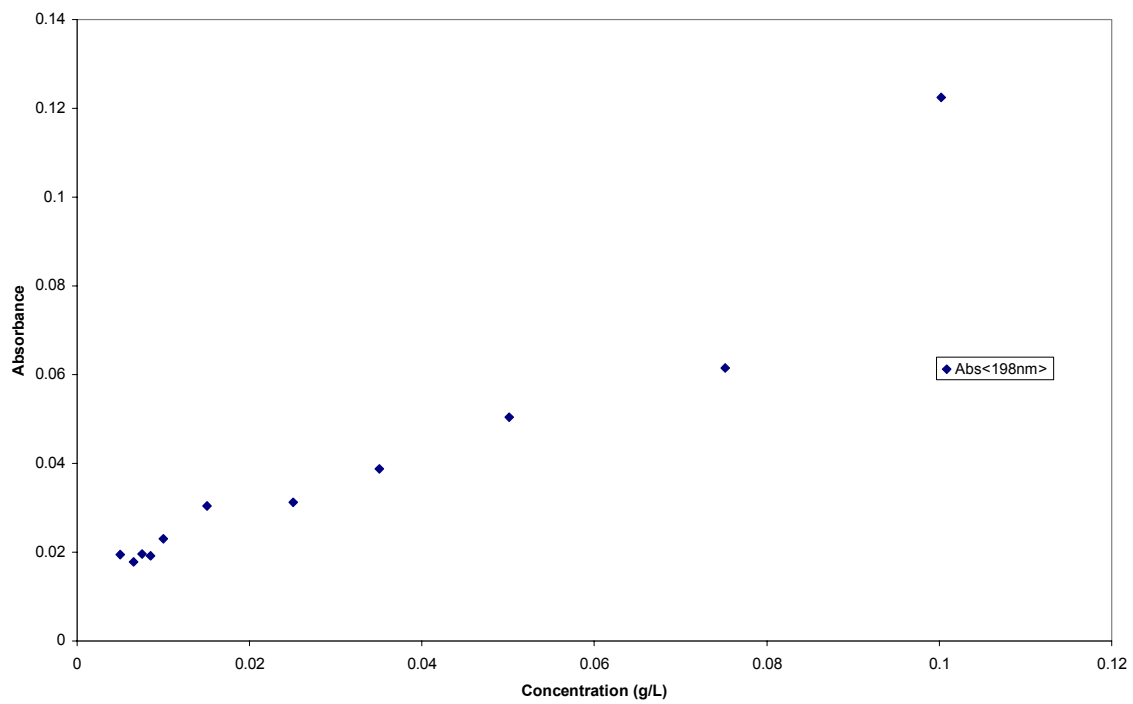


Figure 30

Glutamine: concentration vs. absorbance at 220 nm

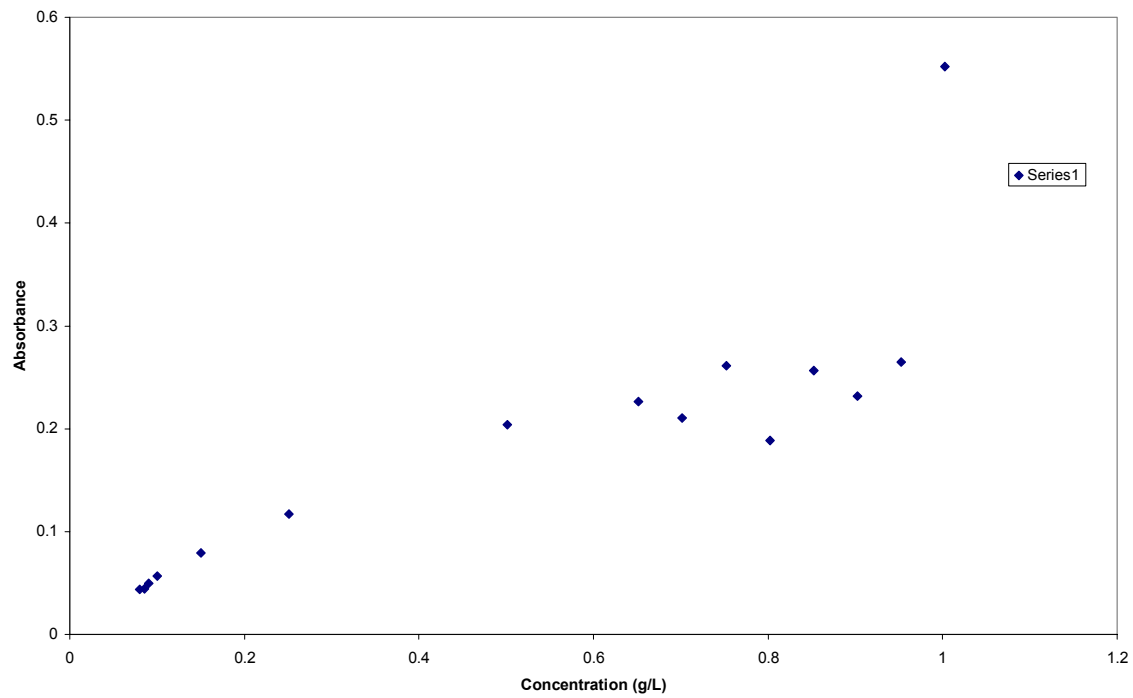


Figure 31

Arginine: concentration vs. absorbance at 205 nm

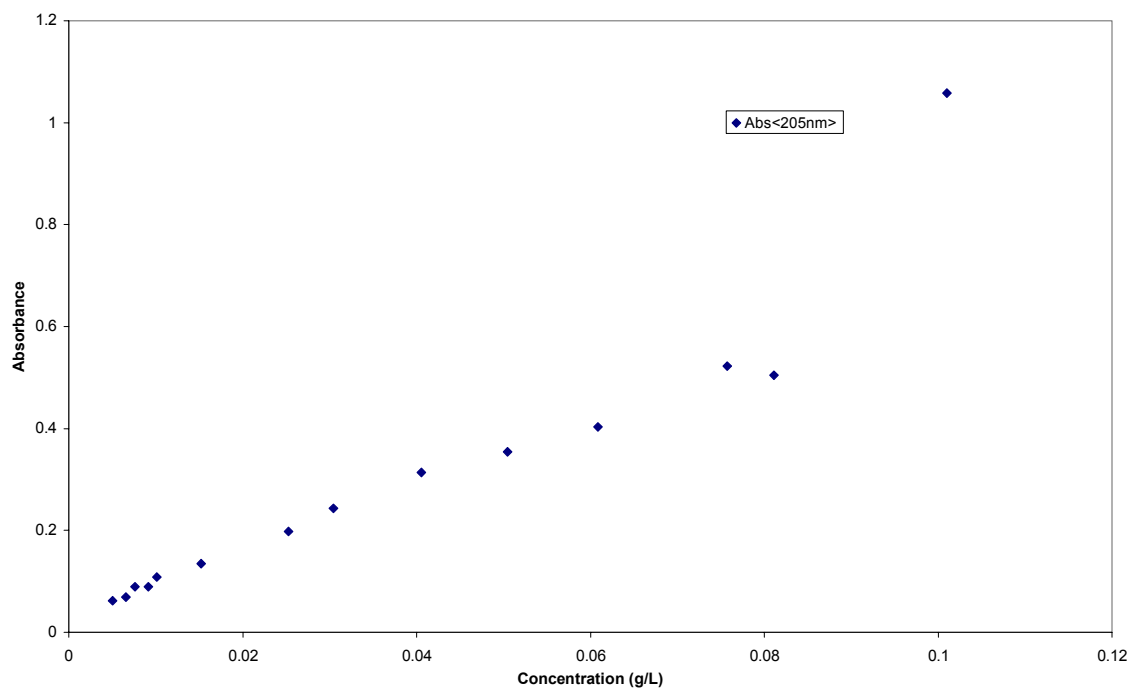


Figure 32

Serine: concentration vs. absorbance at 198nm

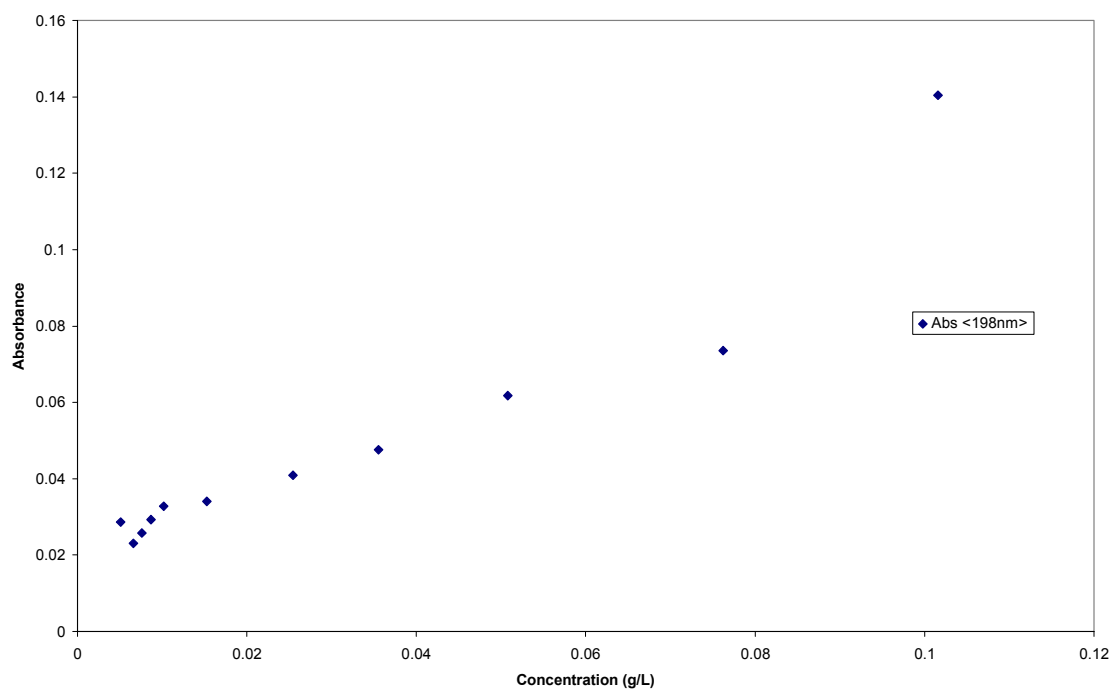


Figure 33

Threonine: concentration vs. absorbance at 190nm

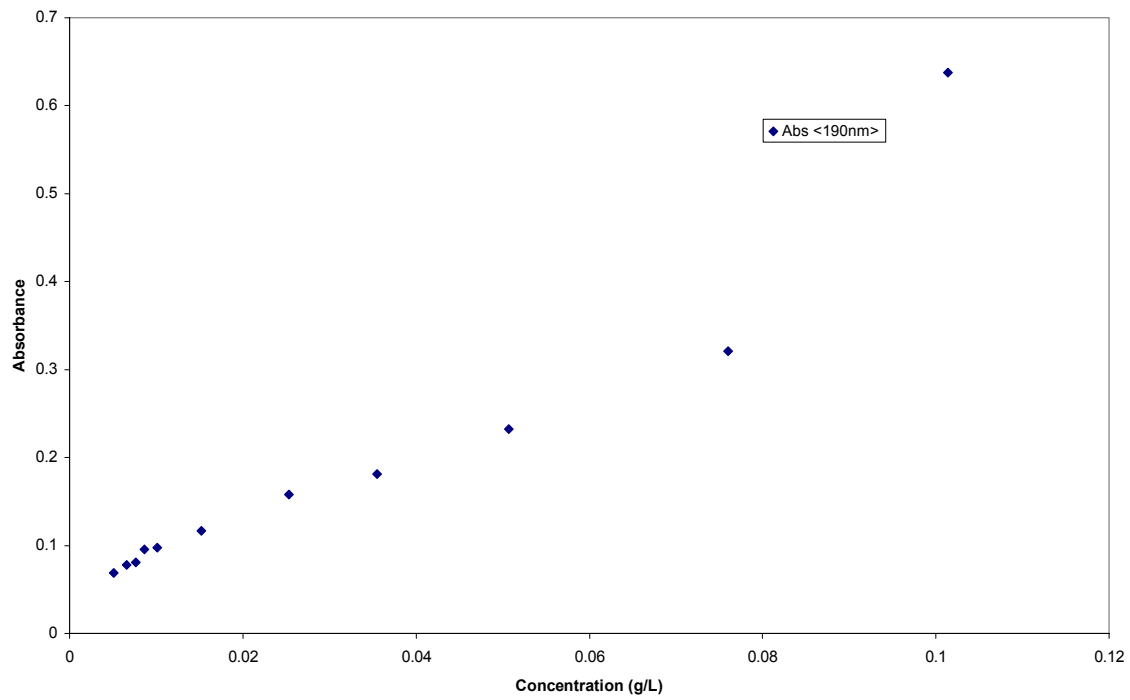


Figure 34

Valine: concentration vs. absorbance at 190nm

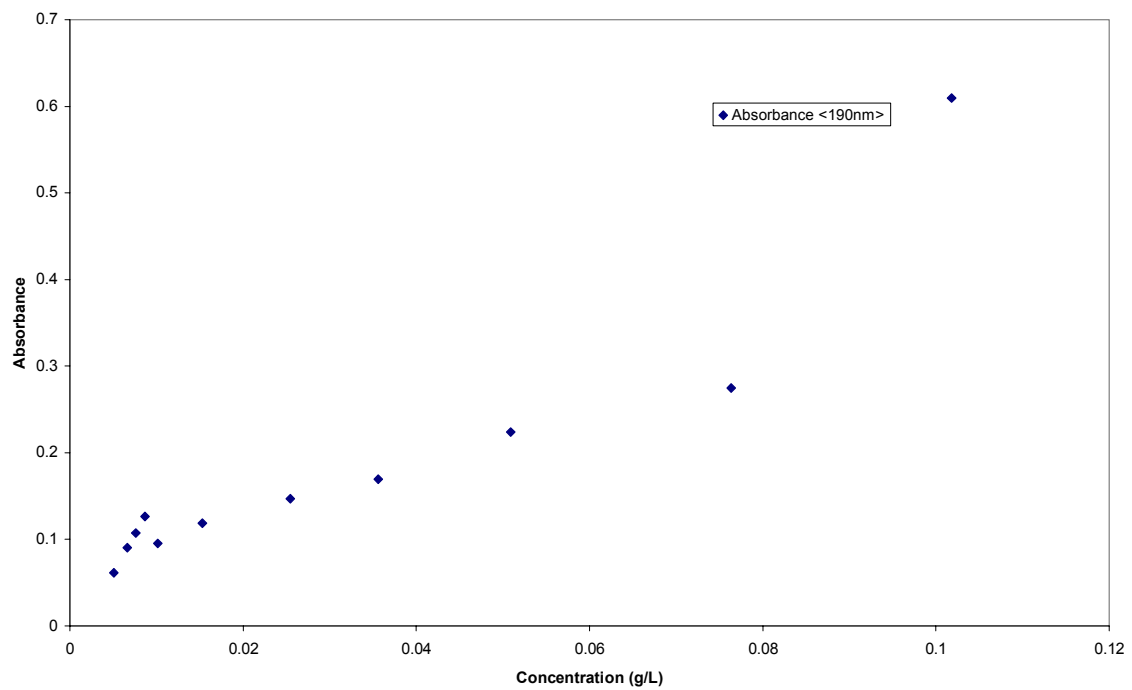


Figure 35

Tryptophan: concentration vs. absorbance at 220 nm

